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Original Article

Superior cervical ganglionectomy alters gut microbiota in rats

Wencui Zhang[1], Yujuan Li[1], Tianning Sun[1], Zhixiao Li[1], Anne Manyande[2], Weiguo Xu[3], Maohui Feng[4], Hongbing Xiang[1]

[1]Department of Anesthesiology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei, China; [2]School of Human and Social Sciences, University of West London, London, UK; [3]Department of Orthopedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei, China; [4]Department of Gastrointestinal Surgery, Wuhan Peritoneal Cancer Clinical Medical Research Center, Zhongnan Hospital of Wuhan University, Hubei Key Laboratory of Tumor Biological Behaviors and Hubei Cancer Clinical Study Center, Wuhan 430071, Hubei, China

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Abstract: The diversity and complexity of sympathetic function highlight the importance of fundamental research. Little is known about the interaction of superior cervical sympathetic ganglion (SCG) and gut microbiota. In this study, the engagement of the sympathetic ganglia with gut microbiota was investigated. Bilateral superior cervical ganglionectomy (SCGx) significantly altered the microbiota composition in rats 14 days post-surgery, and these microbiotas may participate in several biological pathways in the host, suggesting the vital role of the cervical sympathetic ganglion in regulating the microbiome-brain axis, and further confirming that the sympathetic nervous system (SNS) regulates the microbiome-brain axis.

Keywords: Superior cervical sympathetic ganglion, superior cervical ganglionectomy, sympathetic nervous system, gut microbiota

Introduction

Mounting evidence supports a reciprocal relationship between intestinal bacteria and the brain [1]. The gut microbiota comprises miscellaneous microorganisms, including bacteria, viruses and fungi, which populate at the lower gastrointestinal tract [2] and regulate the host functions, such as defense, metabolism and reproduction [3]. The diversity and abundance of each individual's specific members of microbes vary widely, with many factors such as age, host genetics, environment and diet being implicated [4]. The distinctive character of microbiome has been exclusively studied in human diseases, including obesity [5], inflammatory and functional bowel diseases [6, 7] and cardiovascular diseases [8]. Recently, emerging studies have indicated that the gut microbe participates in the pathogenesis of neuropsychiatric diseases, such as autism [9], depression [10], hepatic encephalopathy [11], Parkinson's disease [12] and Alzheimer disease [13]. Multiple tools for manipulating gut microbiota with antibiotics [14], fecal transplantation [15] and germ-free animal models [16] are broadly applied to investigate gut-brain interactions. In addition, considerable evidence demonstrates that gastric microbiota affects cerebral development and function, while the reduced brain-derived neurotropic factor (BDNF) levels [14, 17], abnormal neuropeptide and neurotransmitter levels [17, 18], and altered neuroreceptor expression [18-20] (primarily in the hippocampus) were found in the germ-free and antibiotic-treated animals. Some of these changes correlate with emotional behaviors and cognitive function, implying the vital role of microbial composition in the occurrence of neuropsychological diseases.

The crosstalk between the central nervous system (CNS) and the gut to maintain body homeostasis is achieved by the sympathetic and parasympathetic nervous systems [21, 22], the hypothalamic-pituitary-adrenal (HPA)

axis, and the enteric nervous system (ENS). Combined with the HPA axis, and neural and neuroendocrine signaling, the sympathetic and parasympathetic nervous systems regulate the essential gut duty, e.g., regional motility and permeability, maintenance of epithelial fluid, bile secretion, production of bicarbonates and mucus, and the mucosa immune response [23]. It is now well appreciated that the vagus nerve is the principal regulatory pathway in linking the gut to the brainstem and modulating social behavior [24, 25]. The effect of probiotics and specific metabolites may work through the vagus neural pathway [24, 26-28]. A recent study reported that the bacteria L. reuteri alleviate the social defects in a vagus nerve-dependent tone in mouse models of autism [28]. Likewise, the sympathetic nervous system (SNS) is known to mediate the intestinal mucus layer's physiological properties, consequently regulating the mucosal immune system [29] and microbial composition and behavior alterations [30]. Despite the significance of the vagus nerve in microbiome-brain signaling thus far, the sympathetic branch of autonomic nervous system has not been explored in-depth in the underlying microbiome-gut-brain axis. In addition, the correlation between the SNS and gut microbiota remains to be seen.

The superior cervical ganglion (SCG) is one of the significant components of the SNS with the most traffic branches and special distribution positions. The SCG gives out postganglionic fibers that innervate the pineal gland, hypophysis, carotid body, the iris and eyelid, and participates in the neuroendocrine immune regulation [31]. To excavate the pivotal role of the sympathetic pathway in the microbiome-brain axis, we investigated the fecal microbiome of rats with superior cervical ganglionectomy using 16s rRNA gene sequencing analysis.

Materials and methods

Animals

Twelve male Sprague Dawley (SD) rats (300-400 g; specific pathogen-free grade) were purchased from the Laboratory Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, Hubei, China). Animals were individually housed in a climate-controlled room (temperate of 25±1°C, relative humidity of 50±10%, and a 12 h light/dark cycle) with ad libitum food and water access. All experimental procedures were strictly carried out following with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Institutional Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) (TJH-202102002). Animals were euthanized by overdose of anesthetics after the experiments.

Experimental design

After acclimation for one week, rats were randomly distributed to the following groups: SCGx group (n=6) received bilateral superior cervical ganglionectomy, and faeces were collected from the rats on the seventh day (SCGx-7 group) and the fourteenth day (SCGx-14 group) after surgery. CON group (n=6) received the same surgical procedure without bilateral superior cervical ganglionectomy.

All surgical procedures were conducted with sterile instruments. Rats were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) for all surgeries and body temperature was maintained with a heat regulator. The SCGx surgery was performed as previously described [32]. After anesthetic induction and disinfection, a 2 cm vertical incision was performed at the neck region and salivary glands were exposed. Then, the common carotid artery (CCA) was clearly visible after transected the cranial of the sternomastoid muscle (SMM) and omohyoid muscle (OMH) with blunt forceps. Next the CCA was dissected cranially to locate the carotid bifurcation, and the SCG was just situated behind the carotid bifurcation. Finally, the cell body of the ganglion was gently pulled until their complete avulsion from the nerves chain for collecting the SCG tissue. Absolutely superior cervical ganglionectomy was accomplished by removing the SCG on the contralateral side. After SCG extraction, the incision was closed with 4-0 sutures and compound lidocaine cream was applied topically at the incision to alleviate incision pain. Only exposure of the superior cervical ganglion was performed in the control group. After surgery, rats were put back on a heating pad for recovery and then replaced to their cages. The blepharoptosis was regarded as an indicator of the successful operation.

Fecal collections and 16s rRNA sequencing

Two pellets of faeces were collected from each subject and divided into two parts in a sterilized 1.5-ml EP tube. Fecal samples were snap-frozen on dry ice and stored at -80°C. The 16s rRNA analysis of the fecal samples was performed by OE Biotech Co., Ltd (Shanghai, China). Total genomic DNA was extracted using MagPure Soil DNA LQ kit following the manufacturer's instructions. DNA concentration was verified with NanoDrop and agarose gel. Genomic DNA was amplified using primers targeting the 16S V3-V4 regions. Both primers were coupled with an Illumina sequencing adapter. The PCR products were purified and quantified, and then the concentration was corrected for sequencing performed on an Illumina Miseq PE300 system. The poor-quality reads were screened out and discard using QIIME (ver 1.9.1) software. The clean reads were clustered to generate operational taxonomic units (OTUs) at 97% identity using Vsearch software [33], and all the representative reads were classified with Silva database and PDR classifier via QIIME package [34, 35].

Immunohistochemistry

After removal of SCG, the sections were fixed with 4% paraformaldehyde overnight at 4°C and subsequently dehydrated in 20% sucrose/PBS for 24 h and 30% sucrose/PBS for 48 h and embedded in OCT. 20 um thick serial sections of the ganglion were cut with a Leica cryostat. These sections underwent three 10-minutes washes in PBS and permeabilized with PBST for 30 minutes. After washing, the tissue slices were incubated with 5% bovine serum albumin for 1 h at 37°C. Then sections were incubated at 4°C overnight with the rabbit anti-tyrosine hydroxylase antibody (1:100, catalogue number: E2L6M, Cell Signaling Technology). After three washes in PBS for 10 min per wash, sections were incubated with secondary antibody conjugated with Alexa Fluor 488 (Goat Anti-Rabbit IgG, 1:100) for 2 h at 37°C followed by counterstaining with DAPI. The sections were examined using fluorescence microscope.

Statistical analyses

The relative abundance of gut microbiota and statistical graphs were analyzed using the GraphPad Prism v8.0 package. Data from three groups were analyzed by one-way ANOVA followed by Dunn's post hoc test. The differences in α-diversity (Chao 1 and Shannon index) among CON, SCGx-7 and SCGx-14 groups were analyzed by the Wilcoxon rank sum test. A heat map of Binary-Jaccard distance and the principal coordinates analysis (PCoA) of Binary-Jaccard distance were utilized to visualize the β-diversity. The permutational multivariate analysis of variance analyses of PCoA were performed to observe the overall distribution among groups. The taxa summaries generated in QIIME were reformatted for inputs into LEfSE and linear discriminant analysis (LDA) was used to differentially rank the abundant taxa, followed by Kruskal-Wallis test. For KEGG functional prediction analysis, the functional differences among groups were also analyzed using the PICRUSt software [36] and the results were counted for differences at the class level according to the Kruskal-Wallis algorithm.

All the data are expressed as the mean ± SEM. $P<0.05$ was considered statistically significant.

Result

Verification of surgical performance

After neck dissection and retraction of the mandibular glands, sternocleidomastoid muscle was pulled to the side to expose the CCA. Then the carotid bifurcation was bluntly exposed with the CCA in the cranial direction. The SCG is localized directly underneath the carotid bifurcation. Then, the cell body of the ganglion was gently pulled with microscope forceps until complete avulsion from the sympathetic chain (**Figure 1A**) [37]. The same operation was performed on the contralateral side. Bilateral palpebral ptosis was regarded as an indicator to evaluate the efficacy of the surgery and the rat with bilateral blepharoptosis was apparent after surgery (**Figure 1B**). To demonstrate the sympathetic nature of SCG, the removed structure was stained with antibody against TH, the rate-limiting enzyme in the biosynthesis of catecholamine and a specific marker of SNS [38]. The resected structure presented TH positive immunoreactivity, whereas no distinguishable signal was detected in the absence of primary antibody (**Figure 1C**).

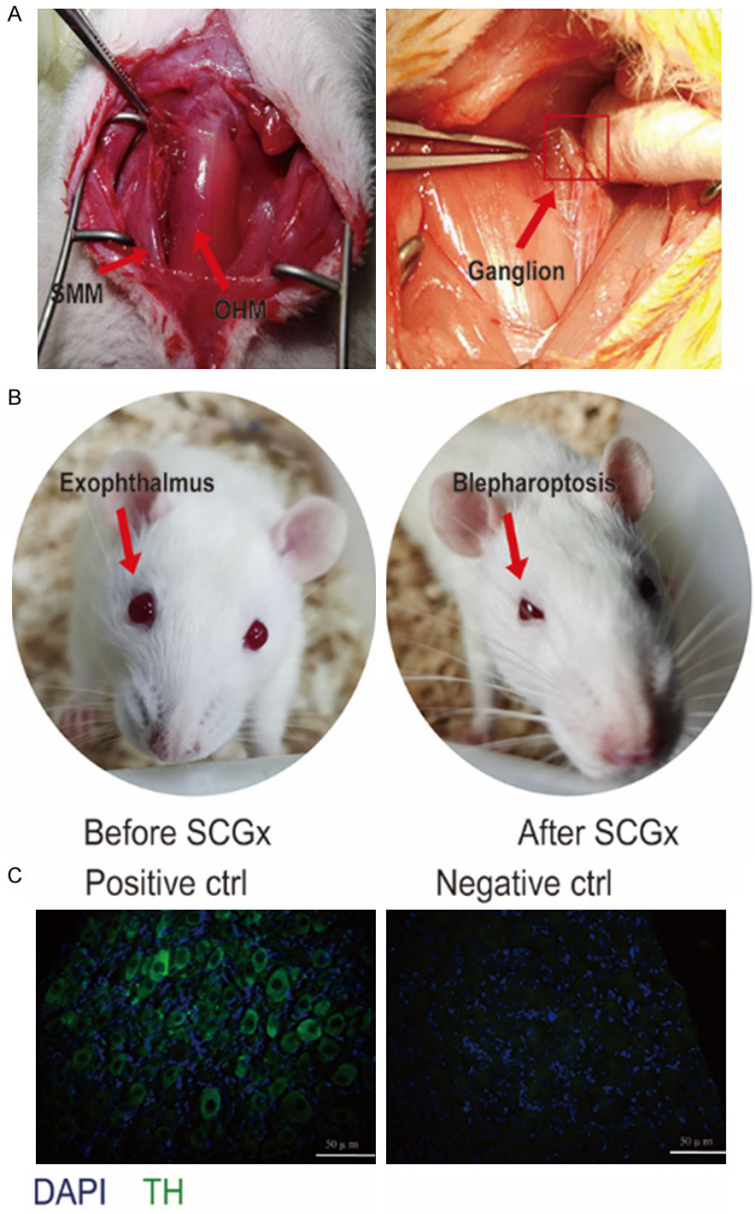

Figure 1. Surgical removal of the SCG in rats and verification by performance. A. The anatomy of the SCG and the neighboring tissues. The glandular tissue and sternomastoid muscle (SMM) were dissected bluntly to make clear the CCA. The SCG was situated behind the carotid bifurcation, and the cell body of the ganglion was gently pulled until their full avulsion from the sympathetic chain. B. The blephar before and after bilateral superior cervical ganglionectomy. C. Immunofluorescence staining of SCG. The same staining protocol was used as a negative control, except that the TH antibody was applied. Tyrosine hydroxylase (TH), scale bar: 50 μm.

Comparison of the gut microbiota composition among the CON, SCGx-7 and SCGx-14 groups

The 16s rRNA sequencing results showed that a total of 12 bacteria at six phylogenetic levels (phylum, class, order, family, genus, and species) were significantly altered among the three groups (**Figure 2A-L**). The relative abundance of 11 bacteria of the SCGx-14 group was significantly altered as compared to the CON group, in which 3 bacteria were decreased (**Figure 2A**, **2I**, **2K**) and 8 were increased (**Figure 2C-H**, **2J**, **2L**). In SCGx-7 groups, there was one significantly increased change (**Figure 2B**) and one significantly declined change (**Figure 2K**), and the remaining ten changes were not significant.

Abundance of the composition of gut microbiota at phylogenetic levels in the CON, SCGx-7 and SCGx-14 groups

Heatmaps of the top 15 gut microbiota at the phylum, class, order, family, genus and species levels in the three groups are shown (**Figure 3A-F**).

alpha-diversity and beta-diversity analysis of the CON, SCGx-7 and SCGx-14 groups

α-diversity depicts the diverseness within a sample, which is mainly relevant to the number of different species, while β-diversity represents the dissimilarity between samples [39]. The Chao1 and Shannon indices are parameters for evaluating the α-diversity. And there was no significant difference in the Chao1 index and Shannon index among the CON, SCGx-7 and SCGx-14 groups (**Figure 4A**, **4B**). Regarding the β-diversity, the principal coordinate analysis (PCoA) plots each sample as a dot in three-dimensional spaces, and demonstrates the difference between groups. It is obvious that the gut microbiome composition profiles in the CON, SCGx-7 and SCGx-14 were clearly separated by PCoA based

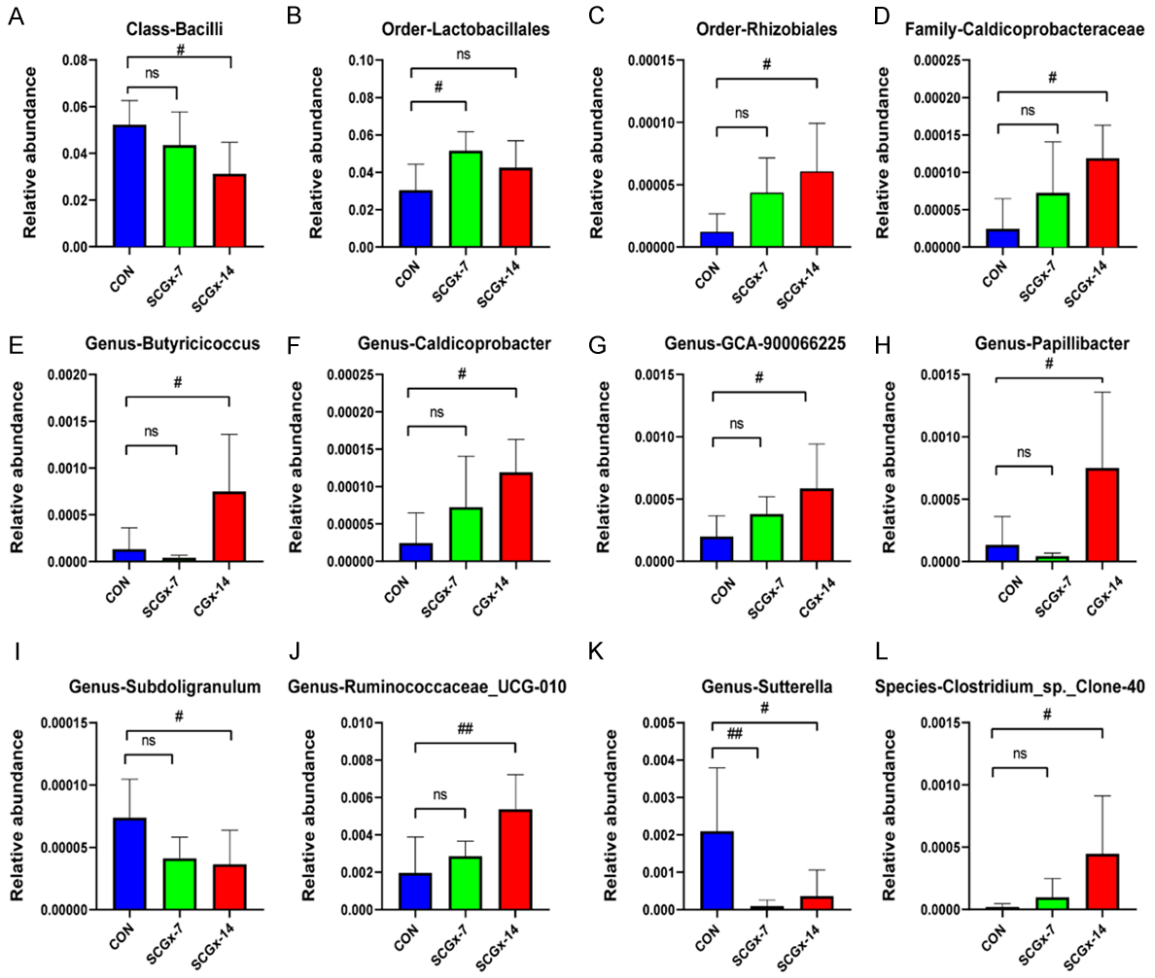

Figure 2. The ANOVA analysis of gut bacteria at differential levels in CON, SCGx-7 and SCGx-14 groups. A. Class-Bacilli (*P*=0.036714). B. Order-Lactobacillales (*P*=0.040188). C. Order-Rhizobiales (*P*=0.030745). D. Family-Caldicoprobacteraceae (*P*=0.023132). E. Genus-Butyricicoccus (*P*=0.009982). F. Genus-Caldicoprobacter (*P*=0.023132). G. Genus-GCA-900066225 (*P*=0.044258). H. Genus-Papillibacter (*P*=0.039208). I. Genus-Subdoligranulum (*P*=0.0485). J. Genus-Ruminococcaceae_UCG-010 (*P*=0.006185). K. Genus-Sutterella (*P*=0.010417). L. Species-Clostridium_sp._Clone-40 (*P*=0.009404). #*P*<0.05, ##*P*<0.01. All three groups were compared by one-way analysis of variance with Dunnett's multiple comparisons test.

on Binary-Jaccard dissimilarity (**Figure 4C-E**). Of note, the 3D PCoA analysis depicts that the dots of the CON group were away from that of the SCGx-7 and SCGx-14 groups, but the dots of the SCGx-7 and SCGx-14 groups were adjacent. In addition, the unweighted pair-group method with arithmetic mean (UPGMA) of PCoA analysis also showed that the CON and the SCGx groups (SCGx-7 and SCGx-14) clustered clearly in their own groups (**Figure 4F**), which demonstrates that there are significant differences in the distribution and composition of species between the CON and SCG groups (SCGx-7 and SCGx-14).

Heatmaps of the compositions of gut bacteria at differential levels in the CON, SCGx-7 and SCGx14 groups

Heatmaps of the compositions of gut bacteria at the order, family, genus, and species levels in the three groups are illustrated (**Figure 5**).

The LEfSe results and prediction of differential microbial functions

To ascertain the especial taxa variably distributed in the three groups, the LEfSe results revealed that 8 taxa were relatively over-represented in the CON group, and 5 taxa and 13

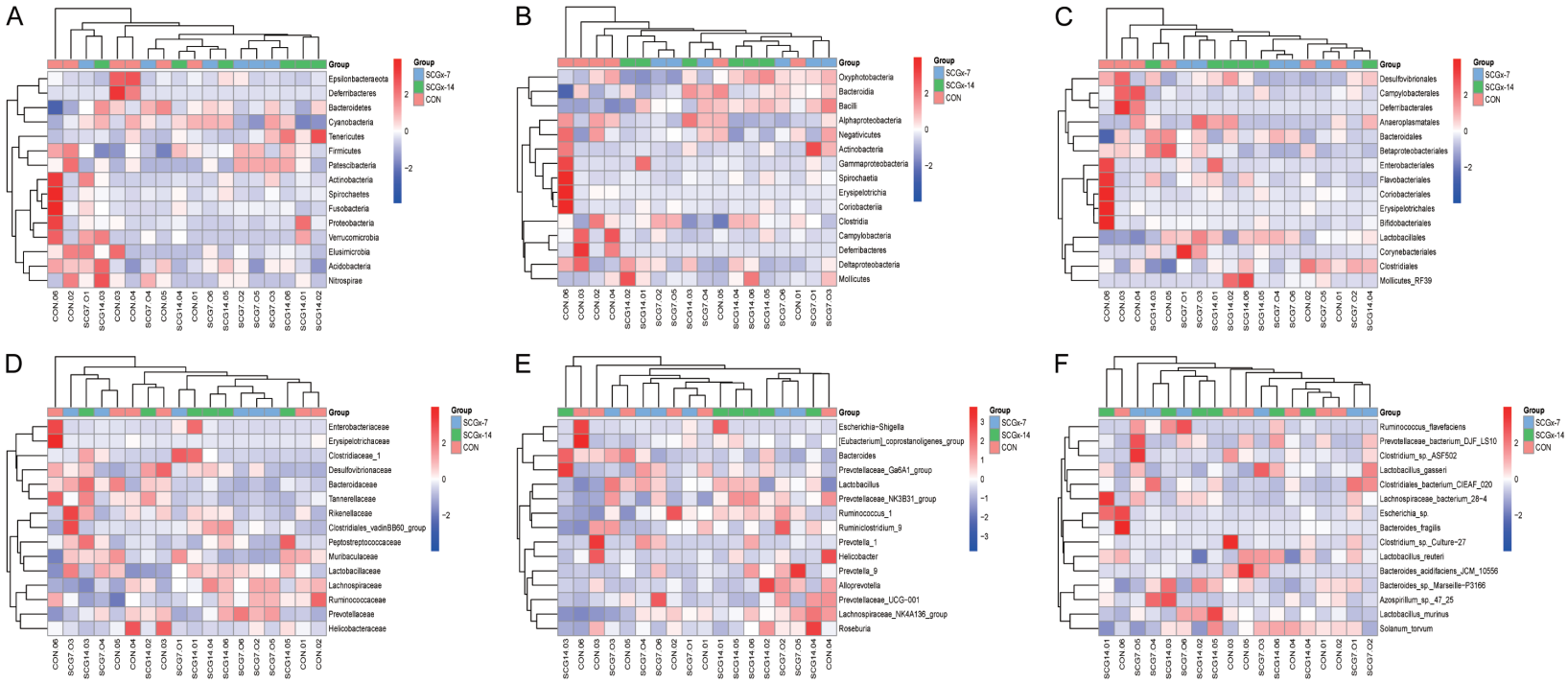

Figure 3. Heatmaps of the abundance of gut bacteria (top 15) at various levels in CON, SCGx-7 and SCGx-14 groups. A. Phylum level. B. Class level. C. Order level. D. Family level. E. Genus level. F. Species level.

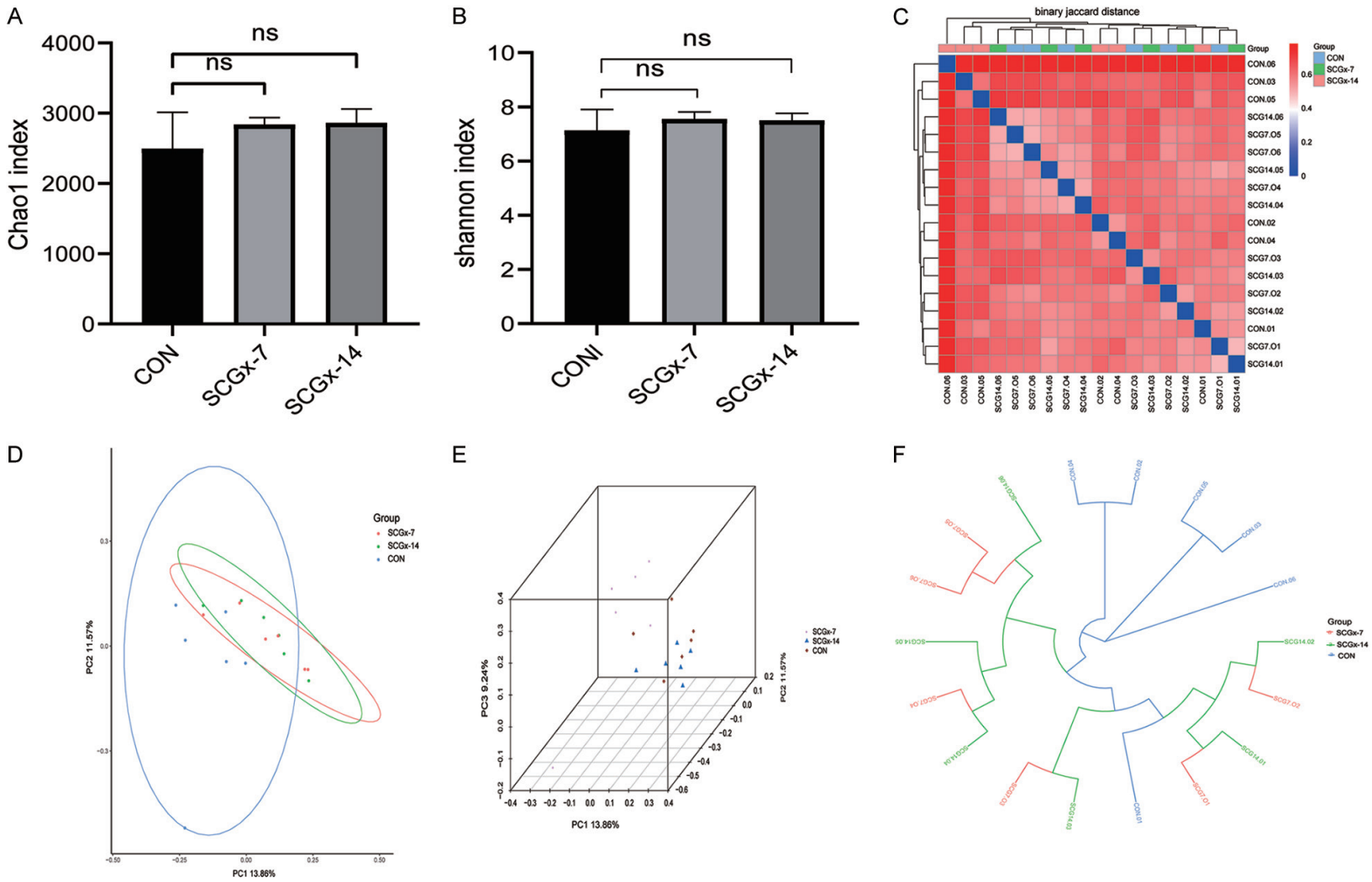

Figure 4. The analysis of alpha-diversity and beta-diversity. A. Chao1 index (P>0.05). B. Shannon index (P>0.05). Data are shown as mean ± SEM (n=6). P values were calculated from the Wilcoxon rank sum test. C. Binary-Jaccard distance. D, E. Binary-Jaccard based Principal component analysis (PCoA) of the three groups. (P<0.05). P was calculated from the permutational multivariate analysis of variance. F. Unweighted UniFrac-based hierarchical clustering analysis of UPGMA in the three groups.

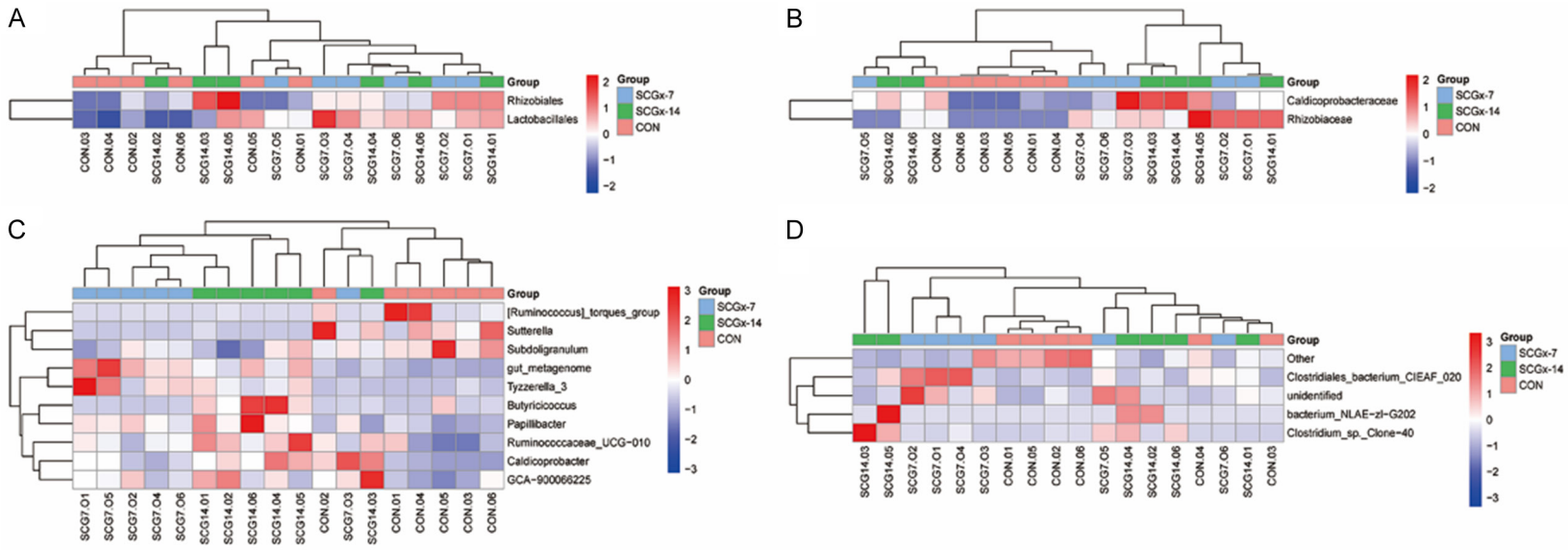

Figure 5. Heatmaps of the compositions of gut bacteria at differential levels in CON, SCGx-7 and SCGx-14 groups. A. Order level. B. Family level. C. Genus level. D. Species level.

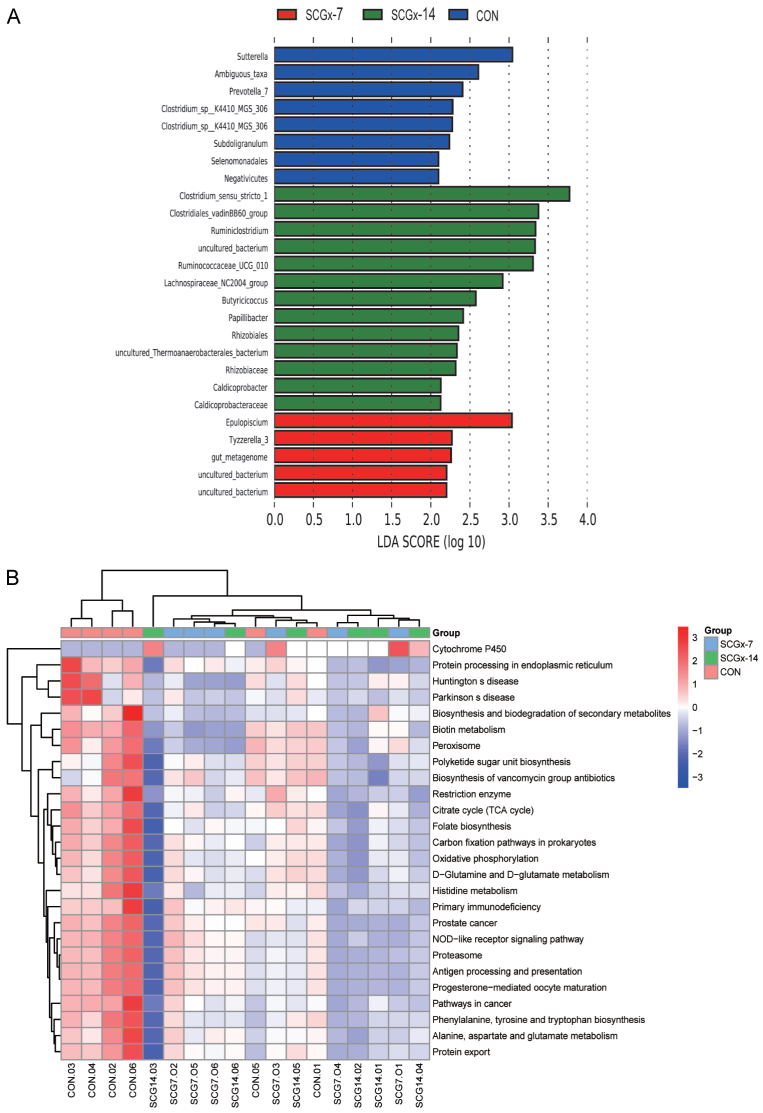

Figure 6. The LEfSe results and KEGG functional prediction analysis. A. The linear discriminant analysis. Red, green and blue bars respectively represent the species with relatively high abundance in the three groups. B. PICRUSt analysis of predicted functional pathway in gut microbiota.

taxa were relatively over-represented respectively in the SCGx-7 and SCGx-14 groups (**Figure 6A**). KEGG functional prediction analysis was performed at class levels. It showed that potential pathways were associated with these differential microbes, including protein processing in the endoplasmic reticulum, neurodegenerative diseases, biosynthesis and biodegradation of secondary metabolites, oxidative phosphorylation, D-glutamine and D-glutamate metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, alanine, aspartate and glutamate metabolism, and the NOD-like receptor signaling pathway (**Figure 6B**).

Discussion

SCGx was introduced in rats in the last century and has been proved to be a valuable model in sympathetic nervous system research [40]. SCG contains thousands of neurons and is located uppermost in the paravertebral sympathetic chain [41]. Surgical removal of the SCG resulted in complete and irreversible sympathetic denervation to all the neuronal cell bodies [31], which disrupted the sympathetic pathway to the eye [42], resulting in blepharoptosis, a phenomenon used as an indicator after SCGx. In the present study, we successfully established SCGx models in rats and clearly found bilateral blepharoptosis post-surgery, especially in the SCGx-14 group. Furthermore, the results of immunofluorescence confirmed the sympathetic nature of the SCG.

Notably, elevated sympathetic activity contributes to the development of hypertension [43, 44]. And the reciprocal communication between the gut microbiota and the brain has been shown to regulate blood pressure by modulating the interaction between the immune and sympathetic nervous systems [45]. Also, considerable evidence has shown the crucial role of microbial dysbiosis in the pathology of hypertension through bacteria metabolic products such as short chain fatty amino acids (SCFAs) [46, 47]. Moreover, SCFA stimulates the paraventricular nucleus (PVN), increasing the sympathetic outflow to the gastrointestinal tract, resulting in a shift in microbial composition. Thus, there is a bidirectional regulation between the sympathetic nervous system and gut flora.

The 16s rRNA gene sequencing is a comparatively cheap and potent tool for uncovering the

microbial community and the link between physiological and pathological characteristics [48-50]. In this study, we tested the altered microbiota composition induced by superior cervical ganglionectomy using 16S rRNA sequencing. And we discovered that the relative abundance of gut bacteria differed markedly between the CON group and SCGx-14 group, and a total of 11 specific gut bacteria were significantly altered between the two groups. At 5 phylogenetic levels, Order-Lactobacillales, Order-Rhizobiales, Family-Caldicoprobacteraceae, Genus-Butyricicoccus, Genus-Caldicoprobacter, Genus-GCA-900066225, Genus-Papillibacter, Genus-Ruminococcaceae_UCG-010, Species-Clostridium_sp._ and Clone-40 were significantly increased in the SCGx-14 group, compared with the CON group. On the contrary, Class-Bacilli, Genus-Subdoligranulum, and Genus-Sutterella were significantly decreased. These results suggest that superior cervical ganglionectomy caused abnormal microbiota composition, implicating the sympathetic pathway in regulating the gut microbiota.

Meanwhile, in our study, the α diversity showed no significant differences among the CON, SCGx-7 and SCGx-14 groups, which suggests that the species and quantity of microbiota did not change in rats that underwent superior cervical ganglionectomy. For the β-diversity, as represented in the PCoA pictures, the dots of the SCGx-7 group and SCGx-14 group were close together, but the dots of both two groups were clearly separated from the CON group. Besides, the circular tree data of UPGMA was in agreement with the PCA and PCoA analysis. Collectively, there was a significant discrepancy in the microbial community diversity between the SCGx and the CON groups.

As previously mentioned, gut microbial community disturbance has been reported to be associated with cardiovascular diseases [51, 52], neurodegenerative disease [12] and obesity [53]. And various pathophysiological mechanisms are involved in the evolvement of these diseases, such as oxidative stress [54], endoplasmic reticulum stress [55], energy metabolism [56], neuroinflammation [57] and unbalanced neurotransmitter [58]. In the present study, we found that the differential microbes were closely related to protein processing in the endoplasmic reticulum, neurodegenerative diseases, biosynthesis and biodegradation of secondary metabolites, oxidative phosphorylation, D-glutamine and D-glutamate metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, alanine, aspartate and glutamate metabolism, and the NOD−like receptor signaling pathway.

Our current study also has several limitations, which should be addressed in the future. Our experimental results just showed the altered microbiota after SCGx, but we did not measure the circulatory and gut NE levels, and the pro-inflammatory cytokines associated with the gut microbiome. The previous study has suggested that the cervical sympathetic trunk takes part in the bidirectional communication between the immune and the sympathetic nervous system [59]. Another question we did not answer is whether cervical sympathetic denervation interferes with gut coeliac-superior mesenteric ganglion (CG-SMG), which specifically project to the gut and directly modulate gut sympathetic activity via the gut-brain circuit [60]. Likewise, the cardiac function might also be affected by SCGx that contributes to the altered microbial composition [61-63]. Hence, we could not clearly clarify the mechanism underlying the altered gut microbiome after superior cervical ganglionectomy. Our data need further studies in the characterization of sympathetic nervous system regulation of gut microbiota and relevant circuit for better understanding the regulation of intestinal motility, enteric immunity and neuropsychiatric disorders related to the gut-brain axis.

In conclusion, these findings revealed that superior cervical ganglionectomy significantly altered the microbiota composition in rats and these microbiotas may participant in several biological pathways in the host. In addition, this study confirmed the vital role of the SNS in regulating the microbiome-brain axis involved with various diseases, many of which are characterized by sympathetic dysregulation. The diversity of metabolites derived from our gut microbiota is quite astounding and also acts locally and systemically in a manner that affects the host physiology in both health and disease conditions. There is an urgent need for further research to identify the exact microbial product regulated by the sympathetic nerve system.

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Disclosure of conflict of interest

None.

Address correspondence to: Hongbing Xiang, Department of Anesthesiology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430074, Hubei Province, China. E-mail: xhbtj2004@163.com; Maohui Feng, Department of Gastrointestinal Surgery, Wuhan Peritoneal Cancer Clinical, Zhongnan Hospital of Wuhan University, Hubei Key Laboratory of Tumor Biological Behaviors and Hubei Cancer Clinical Study Center, Wuhan 430071, Hubei, China. E-mail: Fengmh@whu.edu.cn; Weiguo Xu, Department of Orthopedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430071, Hubei Province, China. E-mail: xuweiguo@tjh.tjmu.edu.cn

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